Somatic Embryogenesis in Chestnut

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Abstract

Somatic embryogenesis is an important biotechnological tool that demonstrates significant benefits when applied to forest tree species; clonal propagation, cryostorage of valuable germoplasm and genetic transformation are among the most promising of its applications. In this chapter, the state of the art of somatic embryogenesis in chestnut (an important economical tree species of the genus *Castanea*) is assessed and discussed. The factors affecting the induction (type of explant, growth conditions, mineral media, plant growth regulators), maintenance and multiplication of the embryogenic cultures (through repetitive embryogenesis) and the maturation and conversion into plants of somatic embryos are described. The latest results achieved on the application of the process on both genetic transformation and cryoconservation of chestnut embryogenic lines are also mentioned.

1 Introduction

In vitro plant regeneration of forest trees (by either organogenesis or somatic embryogenesis) provides tools for cloning superior trees as well as engineering trees with similar efficiency that can be applied to other organisms (Merkle and Dean 2000). There is great interest in applying somatic embryogenesis, not only to mass propagation but also to the development of genetic transformation protocols in forest trees. However, there are several constraints when somatic embryogenesis is applied to these species: in many cases, successful induction only occurs from juvenile tissues (limiting its use for the propagation of mature elite trees), and the quality of the somatic embryos obtained and their conversion rate into plantlets are dependent upon the genotype of the original explant (Stasolla and Yeung 2003). The somatic embryogenesis process is considered to have great potential for sustained clonal propagation, especially when coupled with long-term cryostorage to preserve embryonal tissue juvenility (Park et al. 1998). As somatic embryogenesis is still difficult to achieve in material beyond the seedling stage, cryoconservation precludes genotypes from ageing during the whole selection stage of field-tested, clonally-propagated seed progenies.

Chestnut is an important hardwood species of economical relevance that is found in natural stands, in small groves or grown as nut orchards and coppices throughout the range (Bounous 2002). The *Castanea* genus, belonging to the *Fagaceae* family, is native to the Northern Hemisphere, comprising 13 different species. Since the early 20th century, the populations of *Castanea sativa* Miller (European chestnut) and *C. dentata* Borkhausen (Marshall) (American chestnut) have been devastated by two diseases, ink disease and blight disease, the former caused by the fungi *Phytophthora cambivora* and *Ph. cinnamomi* and the latter by *Cryphonectria parasitica* (Bunrham 1988; Vieitez et al. 1996). To multiply disease-resistant trees or trees selected for specific traits, asexual propagation is required. Difficulties of conventional vegetative propagation by cuttings, air-layering, graft, stooling, etc., have been pointed out (Vieitez et al. 1986); these could be overcome, at least partially, with in vitro tissue culture techniques. Protocols for plant regeneration have been defined for both juvenile and mature tissues, mainly through the proliferation of axillary shoots (Sánchez et al. 1997a,b).

The first report on morphogenetic events associated with somatic embryogenesis in chestnut could probably be dated as early as 1978. Vieitez et al. (1978) showed the differentiation of isolated groups of unorganised sphere-like meristematic pockets in cotyledon explants. Although the work was not properly addressed for the induction of somatic embryos, the structures observed look like the initial stages of what today is known as an embryogenic process. Other authors have subsequently attempted to induce somatic embryogenesis in chestnut, especially in the two species most susceptible to fungal diseases, *C. sativa* and *C. dentata* (Table 1). In this review, the state of the art of chestnut somatic embryogenesis will be described, as well as its potential applications in clonal propagation, genetic transformation and cryoconservation.

2 Culture initiation

To date, there have been few reports on somatic embryogenesis from members of the genus *Castanea*, in spite of their importance. Although considerable effort has been made in recent years, somatic embryogenesis in chestnut has mainly been successfully induced from immature zygotic embryos, as has been the case in many other forest tree species, both Gymnosperms and Angiosperms (Raemakers et al. 1999). The induction of somatic embryos in chestnut from leaf sections published by Corredoira et al. (2003a) opens up new possibilities for induction from mature, selected material.

2.1 Somatic embryogenesis from zygotic embryos

Most of the chestnut embryogenic systems mentioned in Table 1 used both mature and immature zygotic embryos as initial explants. In a first attempt, González et al. (1985) observed the differentiation of embryoids in cotyledon sections excised from mature seeds of a *Castanea sativa* x *C. crenata* hybrid, and cultured on Murashige and Skoog (1962; MS) medium additioned with different concentrations of 2,4-dichorophenoxy acetic acid (2,4-D), either alone or combined with N_6-benzyladenine (BA), kinetin (kin) or zeatin (Z). Histological analyses of the embryogenic tissue showed the presence of globular to cotyledonary somatic embryos (bipolar structures), although the transfer of these structures to a medium without plant growth regulators (PGR) failed to bring about embryoid development into plantlets.

Although Piagnani and Eccher (1990) mentioned somatic embryo formation in one cultivar of *C. sativa*, the first clear report describing the induction of true somatic embryos in chestnut was published by Vieitez et al. (1990). In this study, samples consisting of zygotic embryos excised at different developmental stages were collected from mid-July to mid-October from two ink disease-resistant *Castanea sativa* x *C. crenata* trees. Embryogenic cultures were induced from immature seeds (15-20 mm long) collected 10-12 weeks post-anthesis, cultured on MS medium supplemented with either 0.45 μM 2,4-D plus 4.56 μM Z or 2,4-D (2.26-4.52 μM) with or without 4.49 μM BA for 2 months in darkness. They were then transferred to half-strength MS containing 0.44 μM BA with or without 0.27 μM NAA or 0.25 μM IBA and kept under a 16 h photoperiod (30 μmol $m^{-}2\ s^{-1}$) with 25ºC day/20ºC dark. After 2-3 months, embryogenic cultures consisting of friable yellowish embryogenic tissue or proembryogenic masses (PEMs) formed cotyledonary somatic embryos that were capable of regenerating plants. The overall embryogenic induction capacity was around 2% (Vieitez et al. 1990; Vieitez 1995).

Further experiments were carried out in our laboratory (unpublished results) to better define the induction of the embryogenic system. Plant material was sampled from *Castanea sativa* x *C. crenata* and *C. sativa* trees during the 2001-2003 seasons. Immature zygotic embryos were collected from the last week of August to the third week of September (approximately 10-13 weeks post-anthesis). After sterilization, zygotic embryos were dissected into cotyledon segments and embryonic axes, and were

then cultured for 6 weeks on MS medium supplemented with 3% sucrose, 0.7 % Bacto agar, 500 mg/l casein hydrolisate, 4.52 μM 2,4-D and 0.88 μM BA. After this period, the cultures were transferred to the same basal medium supplemented with BA and NAA at 0.44 and 0.54 μM, respectively. Four weeks later, the explants were transferred to PGR-free basal medium with subsequent monthly subculture to fresh medium. Depending on the genotype, the time required from the initiation of the experiment up to the appearance of the first somatic embryos ranged from 3 to 5 months. Somatic embryos formed on the surface of nodular friable masses induced on the embryonic axis, as well as on the cotyledon pieces, but the induction efficiency was twice as high in the former than in the latter. In the three years studied, the best response was obtained from material collected during the last week of August and the first week of September (10-11 weeks post-anthesis), and the induction rate was clearly influenced by both genotype and year of collection, ranging from 2.2% for the hybrid material collected in 2001, to 10% for *C. sativa* trees collected in 2003. Between 1 and 20 somatic embryos at different stages of development can be obtained from a single explant. Similar results were reported by Sauer and Wilhelm (Ballester et al. 2001) from immature zygotic embryos of *C. sativa* trees collected between 5 and 10 week post-anthesis.

The first report on the induction of somatic embryogenesis in American chestnut was by Merkle et al. (1991), who initiated the cultures from developing ovules and excised immature embryos collected during early and middle stages of fruit development (3-9 weeks post-pollination). Explants were cultured initially for 1 or 2 weeks on Woody Plant Medium (WPM; Lloyd and McCown 1980) containing 1.11 μM BA and either 18.1 μM 2,4-D or 32.2 μM NAA. The competence to initiate somatic embryos was very low, and appeared to depend on the developmental stage of explants, as only ovules collected 6 or 7 weeks post-anthesis produced embryogenic cultures. Ovules which were pulsed on NAA or 2,4-D supplemented medium produced somatic embryos, directly originated from the radicles of the zygotic embryos, and often continued development to the cotyledonary stage; however, explants maintained on auxin-supplemented medium initially generated a nodular growth that resembled proembryogenic masses (PEMs), which formed globular and heart-stage embryos, even while still exposed to auxin, but plantlets were not recorded (Merkle et al. 1991).

A more extensive study was made by Carraway and Merkle (1997) in which immature and mature zygotic embryos were used as explants sampled from 30 American chestnut trees. The effect of three auxins (2,4-D, NAA or 3-indoleacetic acid,

IAA) and two cytokinins (BA or thidiazuron, TDZ) on the embryogenic capacity of seed tissues sampled at different developmental stages was investigated. Across all treatments, genotypes and explant types (12039 explants in total), the embryogenic response was 0.9%. According to these authors, the most efficient induction of embryogenic cultures was achieved from zygotic embryos less than 4 mm in length, and cotyledons smaller than 6 mm^2. Both IAA and 2,4-D induced embryogenic response; however, no embryogenic cultures were recorded on medium containing NAA or TDZ.

Following a similar procedure, Xing et al. (1999) also induced somatic embryogenesis from *C. dentata* developing ovules (4-7 weeks post-anthesis) cultured on an induction medium containing 18.8 µM 2,4-D and 1.11 µM BA. PEMs, identified within 5 weeks after plating, consisted of clusters of globular proembryos attached to the callus surface. An induction frequency of 1.6% was obtained, which did not differ greatly from the values reported by Carraway and Merkle (1997). In *C. sativa* and *C. dentata*, it seems that immature zygotic embryo tissues exhibited a lower competence for somatic embryogenesis induction than those of other related species, such as *Quercus robur* and *Q. suber* (Wilhelm 2000; Hernández et al. 2003).

As occurs in other tree species (Triggiano et al. 1999; Corredoira et al. 2002), the potential of zygotic embryo explants to form embryogenic cultures is influenced by their developmental stage, the developmental window of chestnut responsive material being very narrow. Less mature stages in zygotic embryos were responsive in *C. dentata* in comparison to *C. sativa* or hybrid materials. Another difference to be considered in the embryogenic induction protocols is the culture medium: MS supplemented with 4.52 µM 2,4-D was used for *C. sativa*, whereas WPM plus 13.6 to 18.8 µM 2,4-D was used for American chestnut.

2.2 Somatic embryogenesis from leaf explants

To date, only one report on the induction of somatic embryogenesis from somatic tissues other than the zygotic embryos has been published in chestnut (Table 1). Somatic embryogenesis was initiated from leaf explants excised from stock shoot multiplication cultures of *C. sativa* maintained by sequential subculturing of shoot tips and nodal segments every 4-5 weeks (Corredoira 2002; Corredoira et al. 2003a). The 1-3 uppermost unfurled expanding leaves were excised from 4-week-old shoot cultures, and were cut transversally across the midvein. Proximal (basal) leaf halves were cultured (abaxial side down) on MS medium supplemented with 3% sucrose, 0.7 %

Bacto agar, 500 mg/l casein hydrolisate and different concentrations of NAA (5.37; 10.74; 20 μM) in combination with BA (2.22; 4.44; 8.87 μM). They were maintained in darkness at 25ºC for 6 weeks, and then transferred to the same medium with 0.54 μM NAA and 0.44 μM BA and also maintained in darkness for further 30 days. After this period, leaves were transferred, at monthly intervals, to PGR-free basal medium and kept under a 16-h photoperiod (50-60 μmol m^{-2} s^{-1}) at 25ºC light / 20ºC darkness. Generally, somatic embryos appeared in this medium on the surface of a callus 3-6 months after the culture initiation (Fig. 1A,B), a period that was longer than that observed for induction from zygotic embryo explants. The best results were obtained when leaf explants were initially cultured with 5.37 μM NAA and 4.44 μM BA, with an induction frequency of 1%, a lower value than those obtained from zygotic embryos, which could be expected in a more differentiated tissue, such as that of leaves.

The use of leaf explants excised from shoot cultures to initiate the embryogenic systems offers advantages over the zygotic embryo tissues, as clonal material could be a suitable source of explants for inducing somatic embryogenesis from selected, mature genotypes. In addition, when using leaves from in vitro cultures no sterilization procedure is required, and experiments can be programmed all year around. In contrast to what occurs when somatic embryogenesis is induced from zygotic embryos, 2,4-D was ineffective when applied to leaf sections. The combination of NAA and BA was also used for the induction of somatic embryos from leaf tissues in other *Fagaceae*, including oaks, where embryogenic cultures have been initiated from both juvenile (Cuenca et al. 1999) and adult (Hernández et al. 2003; Toribio et al. 2004) leaf explants.

2.3 Somatic embryo development in the original explants

When using immature zygotic embryos of *C. sativa* or the hybrid material, the initiation of globular embryogenic masses and/or somatic embryos occurred after transfer of explants to PGR-free medium. Nodular masses and somatic embryos appear as translucent white structures that seem to be directly differentiated from embryonic tissue explants (embryonic axes or cotyledon pieces). After isolation of somatic embryos, new embryos generally differentiated from the original explant. It was a common morphology for somatic embryos to have white or pale green cotyledons and a dense, yellowish root pole; fused embryos, embryos with their cotyledons fused together in a cup-like structure, and multiple or anomalous cotyledons were also

produced. Vieitez et al. (1990) reported that nodular embryogenic tissue consisted of nodular masses of small parenchymatic cells, and exhibited areas of great meristematic activity, especially at its periphery, where preglobular and globular stage embryos were also apparent. No vascular tissue was differentiated in these nodular masses, which resembled the proembryogenic masses defined by Halperin (1966). The meristematic areas evolved to develop somatic embryos, which were typically bipolar structures with both shoot and root apices, a closed independent vascular system and no vascular connections with the subyacent embryogenic masses.

The generation of PEMs from American chestnut immature embryo explants was also mentioned by Carraway and Merkle (1997), who reported that after 6 weeks of culture initiation embryogenic cultures began as a mixture of both embryogenic and non-embryogenic callus. To produce cultures with embryogenic potential, 4-5 cycles of visual selection were needed. Approximately 5 months after the first embryogenic tissue was observed, culture lines producing PEMs were established. *C. dentata* embryogenic cultures proliferated as mixtures of embryogenic cell clusters and early cotyledonary stage somatic embryos, and most somatic embryos that differentiated in presence of 2,4-D grew in fused masses with multiple cotyledons; however, the removal of 2,4-D from the culture medium did not preclude the appearance of these anomalous embryos (Carraway and Merkle 1997).

When somatic embryos were originated from leaf tissues of *C. sativa*, the explants initially responded by enlargement followed by a small callus formation, which was mainly differentiated on the leaf cut surfaces. A greenish callus subsequently originated from the midvein, spreading to the rest of the explant. In some cases, translucent globular structures and somatic embryos at various developmental stages began to grow from this callus tissue at different times. The earliest could be seen after one week of transfer to PGR-free medium, although differentiation also occurred after 2-3 months' culture on this medium, which resulted in 3 to 6-month period from culture initiation. The anatomical study (unpublished results) performed on cultured leaf explants showed that they yielded callus tissue comprising parenchymatic cells with vascular elements (Fig. 1C). Certain zones in the periphery of this callus exhibited a gradual disruption of tissue integrity, which gave rise to a friable callus area formed by expanded parenchymatic cells and large intercellular spaces that took on a disaggregating appearance (Fig. 1D). Within this zone, clumps of small densely cytoplasmic cells were differentiated, having a large centrally positioned nucleous with prominent nucleoli, and

accumulation of starch grains (Fig. 1D,E). These characteristics correspond to those displayed by embryogenic cells, whereas the occurrence of embryogenic cell clumps undergoing a series of divisions with a common thick cell wall indicates a probable unicellular origin. Only a small number of these cell clumps continued to develop nodular embryogenic masses that emerged on the disgregating callus surface, and they were generally formed of small vacuolated cells and zones of meristematic cells at the periphery (Fig. 1C,D); neither vascular elements nor starch grains were observed in these nodular masses. Somatic embryos at different developmental stages, including the cotyledonary stage (Fig. 1F), were differentiated from the meristematic areas of the nodular embryogenic masses, which were attached to the callus during initiation but became detached at later stages of development. Embryogenic masses seem to be of unicellular origin, although somatic embryos later originated from these masses appear to be of either unicellular or multicellular origin.

It should be stressed that the generation of nodular embryogenic masses in leaf explants is an indirect process through the formation of an intermediate callus tissue, whereas the PEMs or embryogenic masses differentiate directly from immature zygotic embryo explants.

3 Culture maintenance

In chestnut, the multiplication and maintenance of embryogenic capacity can be carried out via two methods: 1) secondary or repetitive embryogenesis from isolated somatic embryos in torpedo-cotyledonary stages which develop secondary embryos from the root-hypocotyl zone; and 2) subculture of both nodular embryogenic masses and PEMs. The embryogenic masses were produced from the surface of somatic embryos.

Medium-term maintenance of various embryogenic *Castanea sativa* x *C. crenata* culture lines on semi-solid medium has been thoroughly described by Vieitez (1995; 1999) and later updated by Vieitez and Merkle (2005). Essentially, embryogenic lines have been maintained by monthly subculture of PEMs on semi-solid half-strength MS containing 3mM glutamine, 0.91 μM Z, 0.25 μM IBA and 3% sucrose under a 16 h photoperiod at 6-15 μmol m^{-2} s^{-1}. After more than 12 years of repeated subculture on this medium, the production of cotyledonary somatic embryos remains undiminished. The type and concentration of carbon source was also investigated for maintenance of

hybrid embryogenic cultures, where sucrose at 3% was superior to fructose, glucose and maltose, maltose being the least effective (Vieitez 1999).

Corredoira et al. (2003a) also reported the proliferation of embryogenic cultures derived from European chestnut leaf explants, by both secondary embryogenesis and by subculture of nodular embryogenic masses originated from cotyledons of somatic embryos. Secondary embryos were induced by subculturing somatic embryos on proliferation medium consisting of MS mineral salts (half-strength macronutrients) and vitamins supplemented with 3% sucrose, 0.8% Sigma agar, 3mM glutamine, and different concentrations of BA (0.44 and 4.4 μM) and NAA (0.54 and 5.4 μM). As in the hybrid material, low levels of an auxin and a cytokinin were necessary for secondary embryo proliferation. The best results, with a multiplication coefficient of 3.9 (this coefficient was defined as the product of the proportion of explants producing secondary embryos and the mean number of embryos per embryogenic explant), were achieved on medium supplemented with 0.44 μM BA and 0.54 μM NAA (Fig. 2A,B). In addition to secondary embryos, the subcultured primary embryos also began to develop nodular masses from their cotyledons as a form of repetitive embryogenesis. The frequency of nodular clumps producing somatic embryos (Fig. 2C) ranged from 31 to 50 %, with the mean number of embryos per clump ranging from 4.2 to 11.3, the best results (4.6 multiplication coefficient) being obtained with the same PGR combination as for secondary embryogenesis.

The occurrence of both types of repetitive embryogenesis suggests that different cells from the same embryo respond differently to the same culture conditions. The embryonic cells in the hypocotyl-root zone of primary embryos of chestnut are probably embryogenically determined, and a single stimulus for cell division may be sufficient for the formation of secondary embryos. In the case of embryogenic masses originated from cotyledons cells (which are more differentiated), a number of mitotic divisions producing these masses seem to be necessary prior to somatic embryo development. Therefore, direct secondary embryogenesis and indirect proliferation through proembryogenic masses can be considered as two extremes of a continuum (Merkle 1995). A similar process for embryo proliferation was reported for the related species *Q. robur*, in which secondary embryos developed both directly from primary embryos and indirectly from calli originated from cortical tissues (Zegzouti et al. 2001).

When embryo productivity of proliferating cultures reported in Vieitez (1995) and Corredoira et al. (2003a) is compared, it is higher in the former, although the important

effect of the genotype as well as the different origin of the embryogenic systems (zygotic embryos *vs* leaf explants) should be taken into consideration. We have observed that competence for repetitive embryogenesis in different embryogenic lines originated from zygotic embryos of *C. sativa* and hybrid material differs from line to line, highlighting the effect of the genotype on embryo proliferation, an effect that has been well documented in other species (Park et al. 1994; Corredoira et al. 2003b).

The culture of embryogenic masses in liquid medium has also been investigated. Vieitez (1995) established embryogenic cell suspension cultures by transferring proembryogenic masses to liquid medium consisting of MS (half-strength macronutrients) supplemented with 1.13 μM 2,4-D and 0.45 μM BA. Somatic embryos remained arrested at the globular stage, and their further development required the transfer of PEMs to solid maintenance medium. The suspension cultures were allowed to settle for 1 min, then the suspended fraction was discarded, and the settle fraction was resuspended and filtered through a 40 μm size; PEMs were collected and transferred to semi-solid maintenance medium where embryos at all stages of development were observed after 3-4 weeks of culture.

In *C. dentata*, production of secondary embryos was extremely slow and ceased after one or two cycles (Merkle et al. 1991). The maintenance and proliferation of embryogenic cultures has mainly been reported by subculture at monthly intervals of PEMs on semi-solid medium supplemented with 13.56 μM 2,4-D and 1.11 μM BA in the dark (Carraway and Merkle 1997). Suspension cultures were established by inoculating 0.5 g of PEMs in liquid medium with the aforementioned growth regulators, and these were maintained through transfer to fresh liquid medium at 3-week intervals. PEMs proliferated more rapidly in liquid than on semi-solid medium. Production of somatic embryos arrested at the early cotyledonary stage was achieved after removal of PGRs from suspension cultures. Further development of somatic embryos beyond the early cotyledonary stage was obtained when PEMs were transferred to semi-solid medium, where single embryos, clumps of fused somatic embryos and embryos that had multiple cotyledons were observed. In contrast, when PEMs were size-fractionated and transferred to semi-solid PGR-free medium the number of single somatic embryos increased. Addition of charcoal to the basal medium, enhanced the yield and growth of somatic embryos (Carraway and Merkle 1997).

Xing et al. (1999) multiplied American chestnut PEMs on semi-solid medium by subculturing on the initiation medium defined by Merkle et al. (1991) at 2-week intervals and maintaining them in continuous darkness. The development of somatic embryos from PEMs was achieved by transferring them to semi-solid medium supplemented with 0.5 μM BA and 0.5 μM NAA.

4 Embryo maturation and germination

The conversion of somatic embryos into plantlets is currently a limiting step for all chestnut embryogenic systems. It has been shown that in a number of species, low plant recovery rates are due to poor embryo quality and a lack of maturation and desiccation tolerance (Ettienne et al. 1993). In general, maturation of somatic embryos can be achieved through treatments with abscisic acid (ABA) and/or permeating osmotica (high concentrations of sugars, sugar alcohols, amino acids) or non-permeating osmotica (polyethylene glycol (PEG) and dextran) which induce water stress in the culture medium (Lipavská and Konrádová 2004). However, in a number of species, including chestnut, the transfer of previously-matured somatic embryos to a germination medium leads to a poor conversion rate, making it necessary to also apply pregermination treatments, among which we could include cold storage, partial desiccation or the application of gibberellic acid (GA_3), the aim of which is to break the dormancy imposed by ABA and/or osmotic stress.

4.1 Effect of carbohydrates

Carbon source and concentration had a significant effect on the maturation and subsequent germination and conversion ability of *C. sativa* somatic embryos (Corredoira et al. 2003a). In this report, cotyledonary somatic embryos (4-6 mm) were isolated from embryogenic cultures and transferred to various maturation media consisting of PGR-free MS (half strength macronutrients) medium supplemented with sucrose (3 or 6%), maltose (3 or 6%), 3% sucrose + 6% sorbitol or 3% sucrose + 0.5% activated charcoal. After 4 weeks of culture on maturation medium, somatic embryos were transferred to basal medium with 3% sucrose and stored at 4ºC for 2 months, and then cultured for 8 weeks on germination medium (MS with half strength macronutrients and 0.44 μM BA). Plantlet conversion was achieved in embryos matured on media supplemented with 6% sucrose, and with 3% or 6% maltose, whereas mean

shoot length, root length and leaf number of produced plants were not significantly affected by these maturation media, even though higher values were observed after maturation on medium with 6% maltose. Overall, the best results were obtained with 3% maltose-treated embryos, which converted to plants at 6%, in addition to 33% of somatic embryos that developed only shoots (Fig. 2D,E). These shoots were multiplied and rooted following the micropropagation procedure previously described for European chestnut (Sánchez et al. 1997b). Maltose also promoted the somatic embryo maturation of various species (Tremblay and Tremblay 1991; Nørgaard 1997), but its mechanism of action has yet to be elucidated (Lipavská and Konrádová 2004). Nørgaard (1997) assumed that the beneficial effect of maltose in the maturation of *Abies normandiana* somatic embryos may be due to low hexose levels resulting from slow maltose hydrolysis, which limits cell carbon nutrition. Blanc et al. (2002) provided further information that supported the carbohydrate deficit hypothesis to explain the maltose effect.

Carbon source and concentration were also evaluated on the development and maturation of American chestnut somatic embryos. Carraway and Merkle (1997) reported that sugar type had a noticeable influence on number and morphology of cotyledonary stage somatic embryos produced per unit weight of PEMs. Very poor results were obtained with maltose, whereas sucrose promoted development of greater numbers of cotyledonary stage somatic embryo than did fructose, but fructose promoted development of single somatic embryos of normal appearance at higher levels than did sucrose. The contrasting results obtained with maltose, with respect to those achieved in *C. sativa* (Corredoira et al. 2003a), may be due to the genotype or the moment when maltose was applied (cotyledonary embryos in *C. sativa* versus PEMs in *C. dentata*). The preference among carbohydrates has been shown to be species-specific or even cell line-specific (Lipavská and Konrádová 2004). Xing et al. (1999) improved embryo maturation following culture in Gamborg´s B5 medium (Gamborg et al. 1968) supplemented with 0.5 μM BA and 0.5 μM NAA, and with sucrose concentration increased to 6%. Mature embryos then germinated in WPM containing 0.89 μM BA and 0.2% activated charcoal, giving rise to plant conversion, shoot regeneration and rooting rates of 3.3, 6.3 and 12.3%, respectively. The 6.3% of mature embryos developing only shoots could indirectly regenerate plantlets through a micropropagation procedure (Xing et al. 1997). By contrast, Robichaud et al. (2004) reported that sucrose level (3-7.5%) in the maturation medium had no effect on the germination frequency of American

chestnut embryos, suggesting a possible influence of genotype in order to explain the differences obtained regarding previous studies (Carraway and Merkle 1997; Xing et al. 1999).

4.2 Effect of cold storage

As chestnut seeds require cold stratification to germinate, somatic embryos may also need the application of a cold period to break the epicotyl dormancy. In general, this treatment resulted in an overall enhancement of conversion in comparison to previous experiments without chilling. Thus, in hybrid material, plantlet conversion of cold-treated somatic embryos (10-14 weeks at 4ºC) was 18-19% (Vieitez 1995; 1999). The application of a 2-month cold treatment period was also essential to achieve plantlet conversion in *C. sativa* (Corredoira et al. 2003a). The best results, considering both the percentage of somatic embryos developing plants and the percentage of embryos developing only shoots, were obtained with the application of cold storage with or without partial desiccation, giving a total of 41.7 and 38.9% of mature embryos eventually producing plants, respectively. Partial desiccation did not appear to influence the conversion rate.

The effect of a chilling treatment was also investigated by Carraway and Merkle (1997), who concluded that cold storage (8-12 weeks at 4ºC) is necessary for the germination of American chestnut somatic embryos. However, Xing et al. (1999) did not apply this pretreament in their germination experiments.

4.3 Other maturation treatments

Activated charcoal had no positive effect on the germination and plantlet conversion of European chestnut somatic embryos (Corredoira et al. 2003a), whereas in American chestnut it was included in both maturation (Carraway and Merkle 1997; Robichaud et al. 2004) and germination media (Carraway and Merkle 1997; Xing et al. 1999; Robichaud et al. 2004).

The culture of isolated embryos of hybrid material on media supplemented with ABA (0.38-7.45 μM) failed to prevent secondary embryogenesis, and had no effect on their subsequent conversion on MS medium containing 0.92 μM Z and 150 μM Fe-Na-EDTA or on MS with GA_3 at various concentrations (Vieitez 1995). The application of ABA (0.37-37.8 μM) in combination with different gelling agents, as well as the effect

of PEG_{8000} at 2-4% was also evaluated (Vieitez 1999); however, these treatments were very poor in supporting embryo maturation.

As in the case of *Castanea sativa* x *C. crenata* (Vieitez 1995; 1999), addition of ABA to the maturation medium did not increase plantlet conversion of American chestnut somatic embryos (Xing et al. 1999). In a further report, Robichaud et al. (2004) investigated the addition of ABA, PEG_{6000}, and amino acids (glutamine and asparagine) to the maturation medium prior to cold storage for 4 weeks. They found that some of these treatments increased the dry weight/fresh weight ratios and starch content, but did not increase germination ability; only the 25 mM asparagine treatment significantly enhanced the germination rate (14.17 %) and the root length of the germinants.

We also noted that PGRs incorporated into the germination medium affected conversion ability, whereas the somatic embryo size (two classes of 2-5 mm and 6-8 mm) prior to culture on maturation medium did not significantly influence plantlet recovery. The best results (percentage of plantlet conversion and percentage of embryos forming only shoots) were obtained in treatments including 0.44 μM BA with or without auxin (0.54 μM NAA or 0.49 μM IBA), although shoot length, root length and leaf number were enhanced in both PGR-free medium and BA plus IBA supplemented medium.

As has already been mentioned in culture initiation and culture maintenance sections, the genotype is also an important factor influencing germination and plantlet recovery of chestnut somatic embryos (Vieitez 1995; 1999; Xing et al. 1999; Robichaud et al. 2004). Thus, further efforts will be necessary to optimize maturation and germination protocols, in order for them to be applied to a wide range of genotypes.

5 Acclimatization and growth in the field

To date, there is scant information on acclimatization and transfer to soil of plantlets derived from chestnut somatic embryos. Although the results obtained so far indicate that somatic seedlings of *C. sativa* and their hybrids and *C. dentata* can be acclimatized and grown in the field, the number of field-grown plants is currently very low.

Vieitez (1995) transferred somatic plantlets to pots containing a 1:1 mixture of peat moss and quartz sand, and these were kept inside an acclimatization tunnel for hardening. Between 70-80 % of embryo-derived plantlets (116 out 147 for E-431 line and 38 out 52 for E-HV line) survived and resumed growth within 4-8 weeks of

transplantation. Surviving plants were moved to greenhouse conditions and allowed to grow for one year. Some 100 somatic plants were transferred to field, and all of them survived in soil. After two years, their heights ranged from 70 to 110 cm. Surprisingly, many of these plants showed symptoms of precocious maturation, developing male catkins after 3 years, and beginning to regularly bear chestnuts the following year (Fig. 2F). European chestnut plants derived from seeds require around 10-15 years for flowering, although for *C. crenata* and *C. mollisima* this may be earlier, at 3-5 years (Paglietta and Bounous 1979). Precocity of somatic plants is an extremely valuable character which may be useful in breeding programmes.

In American chestnut, Xing et al. (1999) attained acclimatized plants in a growth chamber after transfer of germinated somatic embryos and plantlets micropropagated from shoot-producing embryos to potting mix. Of 20 plantlets acclimatized and grown in a greenhouse, the largest 6 were transferred to the field. These authors also observed that at the end of the second growing season, the four surviving plants averaged 27.3 cm in height in comparison to 61.7 cm achieved by normal seedlings (control). Similar results were recorded by Robichaud et al. (2004), who reported that 6 out of 23 somatic plants survived transfer to potting mix, acclimatization to greenhouse conditions, and transplanting to the field.

6 Applications of chestnut embryogenic cultures

6.1 Genetic transformation

Somatic embryogenesis is not only a promising method for clonal mass propagation, but it is also viewed as a valuable tool for genetic engineering. One of the most important goals in the genetic transformation of trees is to increase resistance to fungal pathogens by transferring genes encoding proteins that are involved in the defence mechanism, such as chitinases (Maynard et al. 1998). The definition of a transformation protocol using marker genes opens up the possibility of applying biotechnological tools to the genetic improvement of chestnut through the development of blight- and/or ink-resistant trees.

Genetic transformation was first attempted by Carraway et al. (1994) and Maynard et al. (1998), who used particle bombardment and *Agrobacterium tumefaciens*, respectively, to transform embryogenic cultures of American chestnut. However, only transgenic cell lines (and no transgenic somatic embryos) were produced. The

development of a reliable and reproducible genetic transformation protocol for European chestnut in which embryogenic cultures initiated from leaf explants were used as the target material was reported by Corredoira et al. (2004a). In this study, a transformation efficiency of 25% was recorded when somatic embryos at the globular to early-torpedo stages were co-cultured for 4 days with *A. tumefaciens* strain EHA105 harbouring the pUbiGUSINT plasmid containing marker genes. Transformation was confirmed by a histochemical β-glucuronidase (GUS) assay (Fig. 2G), PCR and Southern blot analyses for the *uidA* (GUS) and *npt*II (neomycin phosphotransferase II) genes, and germination and plant recovery was achieved from transformed somatic embryos.

6.2 Cryopreservation

Cryopreservation is currently the safest and most cost-effective method for the long-term conservation of species that are vegetatively propagated or which have seeds that are recalcitrant to storage. Chestnut embryogenic cultures are generally maintained by repetitive embryogenesis. To facilitate management of embryogenic lines and limit the risks of somaclonal variation and contamination, as well as to reduce labour and supply costs, cryopreservation may be a reliable alternative. The feasibility of long-term preservation of *C. sativa* germplasm via the cryopreservation of embryogenic cultures has recently been demonstrated by Corredoira et al. (2004b). In this work an embryogenesis resumption level of 68% was obtained by first preculturing 6-8 mg clumps of globular or heart-shaped somatic embryos on medium containing 0.3M sucrose for three days, followed by 60 min application of PVS2 vitrification solution (Sakai et al. 1990) before direct immersion in liquid nitrogen. Successful cryostorage of embryogenic cultures of American chestnut has been achieved using the application of a cryoprotectant/slow-freezing method (Holliday and Merkle 2000), but the vitrification protocol used in European chestnut seems to be both simpler and less expensive.

The cryopreservation procedures developed for chestnut may be applied to the long-term storage of valuable embryogenic lines, such as those derived from selected genotypes or transformed material.

7 Conclusions and future prospects

Chestnut embryogenic cultures were initiated from immature zygotic embryos and leaf explants, although at low induction rates. The most important factors controlling somatic embryogenesis induction are the genotype, the developmental stage of the zygotic embryos, and the type of growth regulators used; an exogenous auxin (either 2,4-D or NAA alone or in combination with a cytokinin) was an essential pre-requisite to initiate chestnut embryogenic tissue. The long-term maintenance of the embryogenic capacity by repetitive embryogenesis makes the continuous supply of somatic embryos possible, as embryogenic cultures can be efficiently multiplied by both secondary embryogenesis and subculture of nodular embryogenic masses or PEMs.

In spite of the numerous maturation and germination treatments assayed, germination and conversion into plantlets is at present a limiting step in the embryogenic process. It should be stressed that cold storage significantly improved plantlet conversion. Although conversion rates are relatively low, an additional higher number of germinating embryos exhibiting only shoot development was also recorded. These shoots could be multiplied and rooted by using micropropagation techniques. Chestnut somatic seedlings can be acclimatized and grown in the field, where they display a normal appearance.

The recent publication (Corredoira et al. 2004a) describing the production of transgenic chestnut plants via somatic embryogenesis offers an additional alternative to the improvement of the species, specifically if plants with increased resistance to fungal diseases are produced. In addition, the combination of somatic embryogenesis and cryoconservation improves the ability to select superior genotypes, allowing the storage of cultures for several years while awaiting the results of field testing.

To optimize the scale-up of plant production, the following aspects of the embryogenic system need to be improved: i) induction from mature material; ii) enhancement of plantlet recovery by investigating embryo synchronization, maturation and germination; iii) ascertainment of genetic fidelity of the regenerants. Most of the information gathered on embryo development in chestnut has been the result of empirical studies. Molecular biology approaches leading to the understanding of the different steps of the embryogenic process in forest trees are scarce, and to the best of our knowledge, no efforts have been addressed in this regard in chestnut. This is probably one of the most promising lines of research for the coming years.

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Legend to Figures

Fig. 1A-F. Induction of *C. sativa* somatic embryos from leaf explants. **A, B** Somatic embryos and nodular embryogenic masses emerging from callus formed on leaf tissues (6.2x). **C** Callus tissue consisting of large parenchymatic cells (ca), and nodular embryogenic masses (em) that arose from the callus (25x). **D** Embryogenic cell clumps (cc) differentiated in the callus tissue which is in contact with embryogenic masses (em). The disruption of callus tissue resulting in the separation of parenchymatic cells at the surface (arrow head) should be noted (62x). **E** Enlarged view of embryogenic cell clumps formed by densely cytoplasmic cells with presence of starch grains (arrow). Note the expanded vacuolated cells of the callus around the embryogenic clumps (247x). **F** Cotyledonary-stage somatic embryo showing shoot and root meristems and an independent vascular system (25x). (Safranin-fast green in C; PAS-naphtol-blue black in D-F).

Fig. 2A-G. Maintenance of embryogenic cultures and plant recovery in European chestnut. **A,B** Embryogenic cultures multiplied by secondary embryogenesis after 6 weeks of culture on proliferation médium (A 3.9x; B 4.9x). **C** Somatic embryos originated from a nodular embryogenic clump explant after 6 weeks of culture on proliferation médium (15.5x). **D,E** Conversion into plantlets (D) and somatic embryo exhibiting only shoot development (E) after 8 weeks of culture on germination medium. **F** Somatic embryo derived trees 12 years after transplanting to soil. **G** GUS-positive somatic embryos transformed with *Agrobacterium tumefaciens* strain/plasmid combination EHA105/p35SGUSINT (4.8x).

Table 1. Summary of somatic embryogenesis studies in *Castanea*

Initial explants	*Induction medium*[a]	*Maintenance medium*[a]	*Maturation medium*[a]
Castanea sativa			
Cotyledon from immature zygotic embryos	½ MS NAA (5.4) or 2,4-D (5.4) + BA (2.2)	–	–
Ovaries/Ovules/Immature zygotic embryos	P24 + 2,4-D (5) + BA (0.5)	P24 + BA (0.89)	P24 + BA (0.89) +Agar 1.1% + cold storage (3 mo)
Leaf sections	MS + BA (4.4) + NAA (5.4)	½ MS + BA (0.44) + NAA (0.54)	½ MS + Maltose 3% (4wk)+ cold storage (2 mo)
Castanea sativa x C. crenata			
Cotyledon from immature zygotic embryos	MS + 2,4-D (0.45-45.3) ± BA, Z or Kin	–	–
Immature zygotic embryos	MS + 2,4-D (0.45) + Z (4.56) or 2,4-D (2.26 – 4.52) ± BA (4.49)	WPM + BA (0.04) or WPM PGR-free	–
Immature zygotic embryos	MS + 2,4-D (0.45) + Z (4.56) or 2,4-D (2.26 – 4.52) ± BA (4.49)	½ MS + Z (0.92) + IBA (0.25)	MS + Z (0.92) (4wk) + cold storage (10-14wk)
Castanea dentata			
Immature zygotic embryos	WPM + BA (1.1) + 2,4-D (18.1) or NAA (32.2)	Induction medium or WPM + BA (1.11) or WPM PGR-free	–
Immature zygotic embryos	WPM + 2,4-D (13.5)	WPM + 2,4-D (13.5) + BA (1.11)	WPM + AC 0.5% (12wk) + cold storage (8-12wk)
Immature zygotic embryos	WPM + 2,4-D (18.1) + BA (1.11)	WPM + 2,4-D (18.1) + BA (1.11)	B_5 + Sac. 6%+BA (0.5) + NAA (0.5)
Immature zygotic embryos	–	WPM + 2,4-D (9.0)	WPM + Sac. 6% + AC 0.1% + Asparagine (25mM) (4wk) + cold storage (4wk)

[a] Quantities in brackets are expressed in μM unless otherwise stated. – not mentioned.
Mineral media: B5 – Gamborg et al. (1968); MS – Murashige and Skoog (1962); P24 - Teasdale (1992); WPM – Woody Plant Medium (Lloyd and McCown, 1980)
Supplements: AC – activated charcoal; BA – N_6 - benzyladenine; 2,4-D – 2,4-dichlorophenoxy acetic acid; IBA – indol-3-butyric acid; Kin – kinetin; NAA – 1-naphthalene acetic acid; Z - zeatin

Table 2 Somatic embryo production (at cotyledonary stage) by secondary embryogenesis and through nodular clumps (50-60 mg) after 6 weeks of culture on different proliferation medium.

Treatment (*μM*)	*Nº of secondary embryos /primary somatic embryo*	*Nº of somatic embryos / nodular clumps*
BA 4.4 + NAA 5.4	3.6 ± 0.6ab	4.2 ± 0.6a
BA 4.4 + NAA 0.54	4.2 ± 0.6abc	5.1 ± 0.7a
BA 0.44 + NAA 5.4	3.1 ± 0.3a	5.2 ± 1.0a
BA 0.44 + NAA 0.54	5.5 ± 0.7c	11.3 ± 1.4b
ANOVA		
F	11.134	11.134
P[a]	0.037*	0.000***
LSD 5%	1.6238	2.7293

Values are means ± standard error of 10 replicates from two experiments with six explants in each replicate.
Within each column, values followed by the same letter are not significantly different at P=0.05 level according to LSD test.

[a]F-test significant at: ***, P<0.001; *, P<0.05

Table 3 Effect of 2 months cold storage pretreatment, germination medium and embryo size on germination of chestnut somatic embryos (line HV-Z2) exhibiting only shoot development or plant conversion. Data were recorded after 8 weeks on germination medium following 4 weeks on 3% maltose maturation medium and subsequent chilling treatment (-/+).

Cold storage	*Germination medium (μM)*	*Shoot only (%)*		*Conversión (shoot+root) (%)*		*Total (%) (conversion+shoot only)*	
		2-5 mm	*6-8 mm*	*2-5 mm*	*6-8 mm*	*2-5 mm*	*6-8 mm*
	BA 0.44	2.0 ± 1.4	6.0 ± 1.4	2.0 ± 1.4	2.0 ± 1.4	4	8
+	**PGR-free**	2.0 ± 1.4	2.0 ± 1.4	22.0 ± 7.1	16.0 ± 5.7	24	18
+	**BA 0.44**	16.0 ± 2.9	18.0 ± 4.3	18.0 ± 1.4	18.0 ± 1.4	34	36
+	**BA 0.44+NAA 0.54**	12.0 ± 2.9	10.0 ± 4.3	22.0 ± 1.4	24.0 ± 5.7	34	34
+	**BA 0.44+IBA 0.49**	16.0 ± 2.9	10.0 ± 1.4	22.0 ± 1.4	22.0 ± 1.4	38	32

Values are means ± standard error of 2 replicates with 25 somatic embryos in each replicate.